Ultrastructural analysis of B. rapa stigmatic papillae to characterize vesicle transport during pollen acceptance

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A crucial step in the process of flowering plant reproduction is the recognition of a compatible pollen grain, as it directly impacts fertility. Recently, it has been demonstrated that vesicle trafficking and the contents of secretory vesicles play an important role in pollen acceptance in the Brassicaceae. Progress has been made in determining this signaling pathway, which includes Exo70A1. Previously, Exo70A1 was demonstrated to function in planta in vesicle delivery at sites of polarized secretion. Recently, it was shown that the exocyst complex is required for the delivery of factors to accept compatible pollen. However, many questions remain in the field as the identification of the receptor(s) that recognize compatible pollen grains through the basal pollen response that activates the transportation of secretory vesicles to the plasma membrane. To understand the process behind vesicle transport during the basal pollen response we have been optimizing our TEM imaging of the pollen-pistil interface. Due to the sensitivity of the pollinated pistil tissue, subtle changes to the TEM reagents are used to fix and embed the tissue and modified TEM techniques are used to successfully and consistently prepare the fragile tissue. To identify the receptor(s) of a compatible pollination and the signaling pathway(s) of the secretory vesicle release, we will perform quantitative proteomics of B. rapa stigmas in compatible and self-incompatible pollinations. We are currently analyzing the data from our preliminary proteomics results to identify likely candidates in the basal pollen response. These candidates will then be characterized in T-DNA knock-out lines to examine their potential role in compatible pollen acceptance. Our experiments will result in building a model for vesicle trafficking in the Brassicaceae.
Unraveling the molecular genetics behind HSI in Primula

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Heteromorphic self-incompatibility (HSI) is one of a variety of breeding systems that have evolved in the Angiosperms to promote outbreeding. In plants possessing an HSI system individuals are one of two or three morphologically distinct mating types and produce flowers that differ in morphology to promote cross-pollination as well as biochemistry in a manner that prevents self-fertilization. As a result of extensive genetic studies on HSI in the genus Primula in the first half of the 20thC, HSI is traditionally been viewed as governed by a single, polygenetic locus (S), with genes residing this S-locus differentially regulating both morphological and biochemical differences between mating types. Though there has been recent progress in mapping the genomic region flanking the S-locus, characterization of the linkage group itself is still missing. Using combined transcriptomic and genomic approaches we have characterized differential gene expression between mating types in Primula vulgaris, identified S-linked BACs and sequenced a substantial (previously uncharacterized) region of the S-locus. The results have identified S-linked genes that are currently being assessed for function in HSI, but suggest overall that our historical view of the S-locus, its content and structure may be in need of revision.
NaPEP2 a novel pistil protein whose function is executed once inside Pollen tubes

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Self-Incompatibility (SI) is a genetically controlled system present in many flowering plants to prevent self-fertilization. SI is controlled by the polymorphic locus S, which encodes both female (S-RNase, pistil expressed) and male (SLF/SFB, pollen, expressed) S-determinants. In S-RNases SI based systems, pollen rejection is triggered by the S-specific interaction between the S-RNase and SLF/SFB. However, other non-S-locus genes are also required for the pollen rejection system to function. These factors are called modifier genes (MGs). To date, only three from the pistil have been identified: HT-B, 120K and NaStEP. Yet, we have genetic evidence in N. alata that in addition to HT-B, 120K and NaStEP there is at least another MG (4936-factor) essential to SI. To identify the 4936-factor, we performed a cDNA library subtractive analysis and identified the NaPEP2 (N. alata Pistil Expressed Protein 2) family, which has homology with Pectin methylesterases inhibitors-related proteins (PMEI-RP). The NaPEP2 family includes three genes: A, B and C. RT-PCR analysis corroborates that NaPEP2 expression is stigma-style specific, because NaPEP2 transcripts are expressed only in mature stigma-style, with no detection in roots, leaves, sepals, petals or anthers. In the pistil, NaPEP2 transcripts are mostly accumulated towards the anthesis, since the highest expression peaks in mature pistils. Immunoanalysis shows that NaPEP2 proteins are accumulated as their mRNA do in mature pistils. Notably, NaPEP2 proteins are higher expressed in SI Nicotiana spp than those SC. Likewise, to determine the NaPEP2 localization; we performed sequential protein extraction from hand-bisected styles using different salt concentration buffers. Results demonstrated NaPEP2 members are indeed secreted and their presence is abundant on the extracellular matrix (ECM) in the pistil transmitting tract (TT). Because pistil proteins involved in SI have their function inside PT, we investigated whether NaPEP2 is also taken up by PTs. To test it, we used longitudinal sections and multiple immunolabeling assays on pollinated and unpollinated N. alata pistils.

Our outcomes give evidence that NaPEP2 localizes on the EMC of the TT; but when pistils are pollinated with both compatible and incompatible pollen NaPEP2 is taken up in by allocated to a vacuole as demonstrated by fluorescence immunolabeling and immunogold approaches. This result is remarkable because proteins such as SRNase and 120K also localize in a PT vacuole that contains the cytotoxic S-RNase activity in compatible PTs. We are currently working on demonstrating if this NaPEP2 colocalizes in the same pollen tube vacuole with the S-RNase and 120K. Likewise, by lost of function experiments we are testing if NaPEP2 is a novel MG playing a role in the SI mechanism in Nicotiana. The current work is presenting a novel pistil-specific family of proteins, that may be playing an essential role in the Nicotiana SI mechanism.
Compatible pollen signaling in *Arabidopsis thaliana*: the proposed role of a stigma-expressed RLCK

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The characteristic dry stigmas in the Brassicaceae means that compatible pollen must first be recognized by stigma papillae for successful germination, but relatively little is known about cellular responses to compatible pollen. In our working model, upon compatible pollen recognition, vesicle trafficking is initiated in the stigmatic papilla towards the plasma membrane under the pollen contact site. This regulated secretion allows for water release and cell wall modifications to mediated pollen hydration, germination, and subsequent pollen tube growth. Our work has shown that EXO70A1, and the other seven predicted exocyst subunits (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO84) are required in the stigma for the acceptance of compatible pollen by regulating pollen hydration and pollen tube entry. As well, we have used a reverse genetics approach to identify candidate signalling proteins for activating this regulated secretion. We identified two stigma-expressed Receptor-Like Cytoplasmic Kinases (RLCKs) which are conserved within the Brassicaceae, supporting their possible involvement in a family-wide compatible pollen recognition pathway. Knockdown of these RLCKs resulted in reduced compatible pollen acceptance, as indicated by reduced seed set, reduced pollen tube penetration of the stigmatic papillae, and reduced pollen hydration. We hypothesize that these RLCKs, as part of an early pollen recognition signalling pathway, function upstream of exocyst-mediated vesicle trafficking. This project represents an exciting first step towards understanding the basal compatible pollen response pathway in the stigmatic papillae.
NaStEP interacts with NaSIPP, a mitochondrial pollen protein of SI Nicotiana species and its role in self-incompatibility

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Most of the angiosperms have developed several mechanisms to prevent self fertilization, like self-incompatibility (SI), which enables the pistil to recognize and reject self-pollen or pollen from related individuals (incompatible cross). SI is controlled by the S- specific interaction between S-RNase (female determinant) and SLF (male determinant). In addition, other non-S-locus linked genes such as HTB, 120K and NaStEP, are essential for SI in Nicotiana. We have been studying NaStEP, which is a stigma specific protein of SI Nicotiana spp. This protein is stored in the vacuole of stigmatic cells and is released upon pollination and re-localized into the stigmatic exudate, after that, it is taken up by pollen tubes (PT). The suppression of NaStEP in transgenic plants disrupts pollen rejection in a S-specific manner. All evidences above suggest that NaStEP functions in pollen rejection could be through its interaction with PT proteins, to test it; we searched PT protein interactors by a yeast-two-hybrid assay. We recovered a cDNA encoding a protein with homology of a mitochondrial phosphate carrier, which we called NaSIPP. Sequence analysis suggested that NaSIPP is homologue to a mitochondrial plant phosphate carrier. By microprojectil bombardment of pollen grains assays was determinate that subcellular localization of NaSIPP is indeed mitochondrial. These data were corroborated by transient expression assays in root and hypocotyl epidermis of Arabidopsis thaliana. To corroborate the NaStEP-NaSIPP interaction in plant cells, we performed a BiFC assay in Arabidopsis seedlings. In addition, we showed that this interaction predominantly occurs in mitochondria. Notably, when NaStEP is co-expressed along with NaSIPP in tobacco PT, its localization pattern changed from a wide cytoplasm distribution to a punctuated pattern, which is another evidence of the physical interaction between both proteins. By RT-PCR expression analysis we give evidence that the NaSIPP transcript is specific and highly abundant accumulated in mature pollen. Notably, this analysis also revealed that NaSIPP messenger is only detectable in SI Nicotiana spp; these expression patterns suggest that NaSIPP is involved in pollen rejection mechanism. To probe it, we performed loss of function assays, using a RNAi::NaSIPP to bombard pollen grains, and the pollen grains were pollinated in Nicotiana pistils. NaSIPP suppression disrupts S-specific pollen rejection. Recent evidence has shown to the mitochondrial phosphate carrier as a component of a permeability transition pore (PTP). This is non-selective pore in the mitochondrial membrane that responds to oxidative stress and high Ca+2 concentration getting an open conformation, which triggers a mitochondrial membrane depolarization that increase mitochondrial permeability. Our results suggest the interaction between NaStEP and NaSIPP could be a potential link in the formation of PTP-like in PT after an incompatible cross and support some reports that the pollen rejection mechanism could involve the program cell death in S- RNase self-incompatibility systems.
Pollen-Pistil Interactions session

Role of electric cues and medium conductivity for pollen tube growth - design of a reproducible experimental platform through Lab-On-Chip

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Pollen tubes are believed to react to a combination of chemical, mechanical, and electrical cues during its journey through the pistil in order to achieve fertilization. Despite extensive work dedicated to the subject it is still not clear how these exogenous guidance signals work or how they are processed internally. Using Lab-on-chip (LOC) technology, we assessed the influence of electric fields on pollen tube growth at the microscale. Microelectrodes were integrated into the LOC in order to enable the application of electric fields in a controlled manner. Simulation of the LOC electrical configuration and characterization of the pollen growth medium conductivity were carried out. DC and AC electric fields were applied to batches of Camellia japonica pollen grains under various conditions. Results show that pollen tube growth is increasingly degraded as the applied DC electric field increases. Furthermore, germination is completely inhibited for sufficiently strong fields. AC electric fields, however, had a restoring effect as growth is promoted as frequency increases beyond 100 mHz, which suggests a significant role of the medium conductivity in enabling cell growth. Interestingly, no sign of pollen tube reorientation was found under any tested condition, weakening the much debated argument for electrotropism in pollen tubes. When exposed to a highly localized field, pollen tubes did not deviate. This work suggests that both strength and frequency of an applied electric field influence pollen tubes, and most likely living cells in general, in a much more subtle way rather than being a macro scale exogenous guiding signal.
Vesicular trafficking and directed secretion regulate cell wall plasticity during rapid cellular growth of pollen tube

Hana Rakusova

The pollen tube grows extremely rapidly involving a highly efficient and exquisitely controlled machinery regulating cell wall assembly at the growing tip. Sustaining the growth process requires the transport of cell wall components and multiple types of enzymes to the growing end of the cell. However, it is poorly understood how intracellular transport contributes to more specific functionalities of the pollen tube such as invasive or targeted growth. The aim of my research is to address the question of how cargo is targeted to specific destinations at the cellular surface of the pollen tube, and how interference of this transport affects the pollen tube's functionality. I investigate processes such as the subcellular targeting and regulation of vesicle transport, and the modulation of cell mechanical properties through exocytosis of cell wall components.

To test the role of intracellular transport in pollen tube functionality, tests assessing the pollen tube's capacity to mechanically penetrate the pistillar tissues are being carried out using a microfluidic platform, the TipChip. This Lab-on-Chip device allows single cell analysis of elongating pollen tubes exposing them to precisely calibrated mechanical obstacles or chemical gradients with simultaneous high resolution and fluorescence imaging. The data reveal how intracellular transport regulates pollen tube invasive growth and the ability of the cell to respond to directional signals - crucial parameters for the pollen tube's ability to perform fertilization in vivo.
Mechanosensitive channel MSL8 regulates osmotic forces during pollen hydration and germination

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Pollen grains experience dramatic changes in cellular water potential as they deliver the male germline to the female gametes. Mature pollen of most angiosperms is desiccated to improve survival during dispersal, and must be rehydrated when it reaches the pistil. Subsequently, pollen must establish sufficient turgor pressure to drive the germination and rapid growth of the pollen tube, which carries the sperm cells to the female gamete. It has been proposed that mechanosensitive ion channels may sense and respond to the mechanical stress resulting from these developmentally programmed osmotic challenges, but the molecular identity and function of these stretch-activated channels has remained unknown.

We have identified and characterized MscS-like 8 (MSL8), a pollen-specific, membrane tension–gated ion channel required for pollen to survive the hypoosmotic shock of rehydration and for full male fertility (Science 350:438-441, 2015). MSL8 negatively regulates pollen germination but is required for cellular integrity during germination and tube growth. MSL8 thus senses and responds to changes in membrane tension associated with pollen hydration and germination. While MSL8 might serve to signal to other cellular components in response to mechanical stress, preliminary data support a role for MSL8 as a direct regulator of osmotic pressure via ion flux. These data further suggest that homologs of bacterial MscS have been repurposed in eukaryotes to function as mechanosensors in multiple developmental and environmental contexts.
Ovule secretomics reveal the importance of post-transcriptional regulation of reproductive proteins.

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From the stigma to the ovary, small secreted proteins play pivotal roles in pollen-pistil interactions. They ensure proper pollen tube (PT) growth (germination, elongation, and guidance) and contribute to the establishment of breeding barriers through species-specific interactions. Among them, ovule-secreted proteins (OSPs) were shown to be of particular interest since they control the very last and most critical steps of pollen-pistil interactions such as PT chemoattraction and reception.

So far, selection of candidate genes for pollen-ovule interactions has mostly relied on transcriptomics, especially by comparing RNA expression levels between sexually functional and non-functional ovules, or by single cell type analyses. Yet, these approaches do not take into account potential post-transcriptional regulation steps, such as the control of translation initiation, protein storage and secretion. To address this issue, we first developed a new tissue-free gravity extraction method (tf-GEM) to collect ovule proteinaceous exudates from the wild potato species Solanum chacoense. This, combined with RNA-seq and mass spectrometry-based proteomics led to the identification of 305 OSPs, of which more than half were classified as ovule-specific. Functional annotation showed that these OSPs are predicted to control a broad range of reproductive functions, in particular PT growth and guidance—with several cysteine-rich peptides, a GABA-transaminase, and a PELPIII-like protein—as well as late-acting self-incompatibility S-RNase. Moreover, label-free protein quantification revealed that, from the transition of slightly immature ovules 2 days before anthesis to mature ovules, during which the capacity to attract PTs is acquired, 106 OSPs exhibited a highly significant increase in secretion levels (+14-fold on average) without being regulated at the mRNA level, emphasizing the importance of post-transcriptional regulation of reproductive proteins.
KAPPA: exploring -omics data to detect and cluster cysteine-rich proteins

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Small, secreted cysteine-rich proteins (CRPs) are known to control key aspects of sexual plant reproduction such as sporophytic self-incompatibility (SCR/SP11), pollen-style interactions (SCA/LTP5, LeSTIG1), as well as pollen tube guidance (TfLUREs, AtLUREs) and reception (EC1, ZmES4) by the female gametophyte. CRPs have a dual sequence structure, involving (i) a highly conserved cysteine spacing pattern – even between distant species – to maintain the disulfide bonds configuration, and (ii) inter-cysteine blocks that can diverge to a large extent. CRPs can thus exhibit a fast evolutive speed while conserving their 3D structure to maintain their function, which explain why several sexual CRPs such as LUREs exhibit a strong species-specificity. Due to this rapid evolution, detection of orthologs and paralogs of already known CRPs with common bioinformatics software has been laborious up to now. In addition, the cysteine backbone itself can evolve and give rise to totally new families of CRPs, but no tool was available so far to detect and cluster CRPs de novo, without relying on a set of reference proteins. KAPPA fills this gap, providing a simple algorithm specifically dedicated to CRPs and other proteins defined by a ‘key aminoacid’. KAPPA precisely and efficiently assesses the similarity of two CRPs using a new quantitative index called k-score, and utilizes this value as a criterion to detect all CRPs similar to a seed reference, or to build de novo groups of similar CRPs thanks to an ad hoc recursive clustering function. Application of KAPPA to detection and clustering of well known CRPs such as defensins or lipid-transfer proteins clearly demonstrated its superiority over classical sequence search methods. Its use on proteomic data available from model organisms will then benefit not only sexual plant reproduction research, but also various areas involving CRPs such as medicine (innate immunity), agronomy (resistance to pathogens), and toxicology (snake venoms).
Flowering plants evolved, to ensure efficient fertilization, siphonogamy by which sperm cells are delivered to the female gametophyte by a pollen tube. This process requires the intimate interaction between the female gametophyte and the pollen tube, and is referred as pollen tube guidance. Pollen tube guidance can be divided into two consecutive stages: the sporophytic stage and the gametophytic stage. In the gametophytic stage, the synergid cells secret small peptides to attract pollen tubes. Pollen tube evolved the corresponding recognition and response mechanism to perceive the female signals. Recently, significant progresses have been made to understand this process. However, the knowledge of the molecular mechanism is still very limited.

To identify new factors involved in pollen tube response, we compared the RNA expression profiles of pollen and pollen tubes and identified a subset of A cell-surface receptors on the pollen tube that perceives female attractant LURE1 in Arabidopsis thaliana.

To investigate their function, the kinase-dead dominant negative (DN) form of MDIS1 was expressed in wild-type plants under the pollen-specific LAT52 promoter. Micropylar targeting of the MDIS1DN-expressing pollen tubes was analyzed under minimal pollination. Meanwhile the micropylar targeting of the pollen tubes of the corresponding knock-out mutants mdis1, mik1 mik2 exhibit decreased micropylar guidance. MDIS1, MIK1 and MIK2 are plasma membrane localized receptor-like kinases with extracellular leucine-rich repeats and an intracellular kinase domain. LURE1 specifically binds the extracellular domain of MDIS1, MIK1 and MIK2, and mdis1, mik1 mik2 pollen tubes respond less sensitively to LURE1. Transformation of AtMDIS1 to the sister species Capsella rubella can partially breakdown the reproductive isolation barrier. Our findings reveal a new mechanism of the male perception of the female attracting signals.
The Arabidopsis Alkaline Ceramidase TOD1 is a Key Turgor Pressure Regulator for Pollen Tube Growth in Pistil

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Turgor pressure plays pivotal roles in the growth and movement of walled cells that make up plants and fungi. However, the molecular mechanisms regulating turgor pressure and the coordination between turgor pressure and the cell wall remodeling for cell growth remain poorly understood. Here, we identified an Arabidopsis mutant, turgor regulation defect 1 (tod1), from Ds transposon insertion lines. The mutant displays an obvious phenotype of shorter siliques and a high frequency of aborted fertilization. Further phenotypic analysis demonstrates that pollen tube growth in pistil is impaired while in vitro growth is not affected. Both genetic complementation experiments and additional T-DNA insertion allele analysis show that the mutant phenotype is caused by the disruption of a putative alkaline phytoceramidase, which is preferentially expressed in pollen tubes and guard cells on siliques. We demonstrate that TOD1 is a Golgi-localized alkaline ceramidase. Stomata assays reveal that abscisic acid (ABA) only promotes pre-opened stomata closing in wild-type siliques, while sphingosine-1-phosphate (S1P) promotes closure of pre-opened stomata both in wild type and tod1. tod1 mutant pollen tubes also show growth retardation inside agarose medium, which can be rescued by application of sphingosine or S1P. Plasmolysis experiment shows that tod1 pollen tubes yield a higher turgor pressure than wild-type pollen tubes. The Venus/ECFP ratio imaging results show that TOD1 mutation caused decreased [Ca2+]cyt in pollen tube. tod1 pollen tubes growth defect can be suppressed by gaut13 mutation. Our data suggest that TOD1 acts conservatively in guard cells and pollen tubes in turgor pressure regulation.
Pollen performance traits reveal pre-zygotic nonrandom mating and interference competition in Arabidopsis thaliana

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The lack of ability to measure pollen performance traits in mixed pollinations has been a major hurdle in understanding the mechanisms of differential success of compatible pollen donors. In previous work we demonstrated that nonrandom mating between two accessions of Arabidopsis thaliana, Columbia (Col) and Landsberg (Ler), is mediated by the male genotype. Despite these genetic insights, it was unclear at what stage of reproduction these genes were acting. We developed an experimental strategy that allows us to differentiate different pollen populations in mixed pollinations to ask (1) what pollen performance traits differ between Col and Ler accessions that direct nonrandom mating? And (2) is there evidence of interference competition?

We use genetically marked pollen that can be visualized colorimetrically to quantify pollen performance of single populations of pollen in mixed pollinations. We use this and other assays to measure pollen viability, germination, tube growth, patterns of fertilization and seed abortion. Finally, we assessed interference competition.

In mixed pollinations, Col pollen sires significantly more seeds than Ler pollen. Col pollen display higher pollen viability, faster and greater pollen germination, and faster pollen tube growth. We saw no evidence of nonrandom seed abortion. Finally, we found interference competition occurs in mixed pollinations.

This information allows us, for the first time, to construct a model for pollen competition that incorporates all the major pollen performance traits. The lack of differences in post-zygotic processes indicates that nonrandom mating in Arabidopsis thaliana is pre-zygotic, due to differential pollen germination and pollen tube growth rates. Finally, we unambiguously demonstrate the existence of interference competition.
Testing SI x SC rule in the tomato clade (Solanum section Lycopersicon) and the role of a low activity S-RNase in interspecific reproductive barriers

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Studying the nature of interspecific reproductive barriers (IRBs) among close relatives can provide insight into how species maintain their integrity. Interspecific pollen rejection frequently displays the SI x SC rule, in which crosses between self-incompatible (SI) species and self-compatible species (SC) are successful in one direction but not the other, resulting in unilateral incompatibility (UI). This implies that SI mechanisms may be involved in IRB systems. Pollen-pistil interactions were assessed in interspecific crosses designed to test the constancy of the SI x SC rule in the tomato clade (Solanum sect. Lycopersicon). Generally, the SI x SC rule was followed in crosses at the species level, but there were exceptions with more recently evolved SC populations. In addition I investigated whether S-RNase protein (SI pistil factor) is involved in IRBs in the SC wild species Solanum neorickii. S. neorickii populations located at the species northern and southern margins reject interspecific pollen and express a low activity S-RNase protein. In contrast, S. neorickii in the middle of the species range does not reject interspecific pollen and lacks expression of the S-RNase. In F2 plants of inter-population hybrids, it was observed that individuals that reject pollen tubes also express S-RNase. However, we also observed individuals that express S-RNase but do not reject interspecific pollen tubes. These findings suggest that a low activity S-RNase, although insufficient for SI, can act in IRBs, and further that S-RNase is necessary but not sufficient to reject interspecific pollen tubes in S. neorickii.
An endoplasmic reticulum-localized membrane protein is involved in pollen tube growth

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The distinct reproductive structure and mechanism were evolved during the plant migration from ocean to the land. The loss of sperm mobility, emerging of multi-cellular gametophyte and siphonogamy attributed angiosperm the dominant terrestrial plants. Siphonogamy, a mechanism that male gametophytes (pollen) germinate a pollen tube to deliver the two immotile sperm cells to the female gametophyte for fertilization, enables angiosperm plant to complete fertilization without water and requires an active communication of the male gametophyte with the female gametophyte and maternal tissues, a process that is called the pollen tube guidance.

The pollen tube responds to the signal emitted from the embryo sac to precisely deliver the sperm cells. POD1, as an ER-localized protein interacting with CRT3, is essential for micropylar pollen tube response. But the molecular basis of POD1 is still unknown. Through biochemical strategy, we identified the POD1 interacting partner ECO. ECO is a member of integral membrane protein family. eco mutant exhibits reduced pollen tube growth and guidance, leading to male sterility. We analyzed the subcellular localization of ECO and its genetic relationship with POD1. This study also provides insight into the function of endoplasmic reticulum in intercellular signaling.
Pollen-Pistil Interactions session

Dynamics of reproductive barriers in wild tomato species

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While the molecular basis of self-incompatibility (SI) is well-studied, far less is known about the nature of reproductive barriers between species, and how these barriers arise as populations diverge during speciation. The tomato clade (Solanum section Lycopersicon) is particularly amenable to the study of reproductive barriers (RB), as it contains both self-compatible (SC) and SI species, with numerous examples of two or more species growing in sympatry. Members of the Solanaceae exhibit gametophytic SI, which involves genes at the S-locus (pistil S-RNase and pollen F-box) as well as non-S-locus modifier genes. In interspecific crosses, the ‘SI x SC rule’ is generally followed, where pollen tubes from SI species are accepted by SC species, but the reciprocal cross fails. To investigate interspecific RBs in natural populations of wild tomato, we surveyed 12 sympatric sites in which species have been observed to co-flower. Strong post-pollination, pre-zygotic barriers were detected that followed the SI x SC rule. In the absence of this pollen-pistil barrier, hybrid fruits were formed, but in most cases strong post-zygotic barriers prevented normal seed development. Viable hybrid seeds were formed between sympatric pairs in only three out of 28 interspecific crosses, and we are conducting additional analyses to determine if other post-zygotic barriers, such as hybrid necrosis or lethality, exist in these cases. To better understand how changes in mating system impact interspecific and inter-population interactions, we characterized the dynamics of RBs in Solanum habrochaites, which has undergone SI to SC transitions at the species margins. We found that loss of S-RNase was associated with SC in most marginal northern populations, and that subsequent loss of additional pistil factors weakened interspecific barriers. In addition, pollen from a subset of these northern SC populations was rejected by pistils of central SI populations, demonstrating the formation of an inter-population RB due to loss of pollen side factors. This suggests that mating system transitions are followed by additional loss of function mutations that influence both interspecific and inter-population RBs, and that these mutations can limit gene flow in diverging lineages.
Biochemical events associated with pollen-stigma interaction in sunflower

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Present investigations aim at presenting a detailed structural and biochemical analyses of pollen and stigma in sunflower (Helianthus annuus L.) in order to understand the process of initial events of pollen-stigma interaction. The exine pattern is of ‘Helianthoid type’ which allows easy communication between the pollen surface and the caveae, thus facilitating the exchange of water and physiologically active substances. Sunflower stigma bears extracellular secretions in the basal region of the papillae. The plasmomeric connections between papillae and the transmitting tissue indicate symplastic transport of various metabolites to the papillae and stigma surface. In the tryphine fraction of sunflower pollen grains, unsaturated and saturated free fatty acids make up to 55 and 30% of the total fatty acid content, respectively. The low lipid content in the stigma (2.7% of fresh weight) could be attributed to its semidry nature and absence of apparent stigmatic secretions. Six isoforms of acyl ester hydrolase activity in pollen grains and eight isoforms in the stigma have been detected. Lipase activity has been localized on the pollen coat which might be involved in the degradation of cuticle present on the surface of stigma. The nature of lipases reported in stigma, is yet to be elucidated and it is considerably less on the stigmatic papillae as compared to that in tryphine. These observations indicate the possible interaction of lipase localized on the tryphine zone, with triacylglycerides (TAGs) present on the stigmatic papillae, during pollen-stigma interaction.

Keywords: Pollen-stigma interaction, sunflower, lipase, fatty acyl ester hydrolase, lipids.

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A class of small cysteine-rich pollen coat proteins are important regulators of pollen hydration in A. thaliana

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The early stages of pollination in angiosperms involve multiple phases of interaction between male and female reproductive tissues. The establishment of the pollen-stigma interaction is proposed to involve a basal compatibility system that enables compatible pollen to be recognised by the receptive stigma. Divergence of components involved in this system could facilitate the establishment of prezygotic breeding barriers that would limit wasted mating opportunities, restrict interspecies gene flow and contribute to reproductive isolation. A diverse family of small secreted cysteine-rich proteins (CRPs) has been identified as having multiple roles in plant reproduction. CRPs found in the pollen coat of members of the Brassicaceae, the pollen coat proteins (PCPs), are emerging as important regulators of the pollen-stigma interaction. One class of PCPs isolated from the pollen coat of Brassica oleracea, the PCP-Bs, have previously been described, but their function was unknown. In this study, four putative Arabidopsis thaliana PCP-B-encoding genes were identified, determined to be gametophytically expressed during the late stages of pollen development and confirmed as pollen coat proteins. Bioassays utilising single and multiple pcp-b gene knockouts revealed that AtPCP-Bs function in the early stages of pollination. Pollen morphology was unaffected in pcp-b lines, however mutant pollen grains showed striking defects in pollen hydration, delays in pollen tube emergence, as well as weakened anchoring of pollen grains to the stigma surface. This evidence suggests that AtPCP-Bs, are important components of the basal compatibility system, establishing a molecular dialogue between compatible pollen grains and the stigma. Ongoing work focuses on identifying stigmatic targets for the AtPCP-Bs. This study sheds new light on the biological and evolutionary significance of CRPs in plant reproductive signaling.
Sequence characterization of stylar arabinogalactan genes from distantly related *Nicotiana* species as determinants of pollen tube growth

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The style of *Nicotiana tabacum* controls pollen tube growth in part by secreting arabinogalactan proteins (AGPs) into the extracellular matrix, the path for pollen tubes. The stylar AGPs include: the Class III Pistil-Specific Extensin-Like protein (PELPIII) essential for *N. tabacum* prezygotic interspecific incompatibility with *N. obtusifolia* and *N. repanda*; the 120 kDa protein (120K) required for *N. alata* self-incompatibility and the Transmitting Tissue Specific protein (TTS) that promotes self-pollen tube growth in *N. tabacum*. The glycosylation pattern of stylar AGPs is significant in defining AGP-protein interactions. We hypothesized that AGP polymorphisms among *Nicotiana* spp. is important for regulating pollen tube growth within and among species. The cDNAs of PELPIII and TTS were sequenced from distantly related *Nicotiana* spp. and genomic data from three *N. tabacum* varieties were analyzed in search for AGP related genes. The N terminal domain (NTD) has a high level of insertion-deletion (INDEL) polymorphisms among species and among AGPs. Most of the NTD was predicted to be an intrinsically disordered and is proline-/hydroxyproline-rich. Predicted O-glycosylation sites show variable patterns among *Nicotiana* species, however no specific O-glycosylation pattern was recognized for PELPIII, TTS or 120K proteins. The C terminal domain (CTD) of the stylar AGPs is cysteine-rich, predicted to have a globular structure, contains multiple predicted beta-sheets and is conserved among species and among stylar AGPs. The cysteine pattern resembled that found in the Pollen Ole e I superfamily. A new AGP gene, N.tabAGP, was identified. The N.tabAGP gene and its predicted protein structure were conserved with that of other stylar AGPs. The TTS and N.tabAGP had the greatest amino acid and O-glycosylation pattern conservation among species relative to the PELPIII and 120K genes. It was determined that the PELPIII gene underwent negative selection. It is possible that the high level of INDEL polymorphisms and variable O-glycosylation patterns observed in PELPIII are responsible for the variation of *Nicotiana* spp. pollen tube growth rate in *N. tabacum* styles. High conservation of the CTD among *Nicotiana* spp. and the four AGPs indicates a common and biologically important function for this domain. We hypothesize that the stylar AGPs have a common origin, with initial intron insertion followed by two gene duplication events. Further characterization of the newly identified N.tabAGP and functional analysis of known stylar AGPs will define their role in regulation of pollen tube growth.
Forces Generated by Polar Cell Growth within a 3D Matrix

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We started to develop an assay to investigate how mechanical and environmental signals influence pollen tube behavior in a 3D matrix. By using automated time lapse imaging of pollen tubes growing through media of varying stiffness we aim to investigate the forces generated by the growing cell and to study how the surrounding matrix influences cell growth. Various growth parameters (velocity, direction, pollen tube dimensions) will be analyzed using the imaging analysis tool “ClickPoints”. Software solutions to investigate further parameters (cytoskeleton and vesicle dynamics and cell wall size) are planned. Our new assay system allows us to study the cellular growth behavior by imitating the resistance cells usually face during growth through tissue or soil. Ultimately we will apply this method to screen for mutant phenotypes in polar growing cell types.
Pollen-Pistil Interactions session

Role of Ca2+ circuits and Ca2+-Dependent Protein Kinases in Pollen Tube Tip Growth and Fertilization

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In plants, Ca2+ signals are implicated in all aspects of the life cycle, including pollen tube tip growth and fertilization. Ca2+ transients are created by regulating influx of Ca2+ through ion channels, and efflux through Ca2+ pumps and co-transporters. This regulated flow of Ca2+ creates a Ca2+ circuit. In Arabidopsis, three different Ca2+ circuits have been defined by the presence of ACAs (autoinhibited Ca2+-ATPase pumps) at the PM (plasma membrane), ER (endoplasmic reticulum), and tonoplast. In pollen, critical functions have been identified for PM- and ER-located Ca2+ circuits using genetic knockouts of ACAs (1, 2, 7 and 9) and CNGCs (cyclic nucleotide gated channels 7, 8, 18, and 16). The Ca2+ signals created by Ca2+ circuits are decoded by many different proteins, including Ca2+-dependent protein kinases (CPKs). In Arabidopsis, there are 34 CPKs, 12 of which show significant expression in pollen. Isoforms CPK17 and 34 are located at the PM. A double gene knockout (KO) of cpk17/34 results in a near-sterile pollen-autonomous phenotype in which pollen transmission is reduced by ~350-fold. While cpk17/34 pollen tubes are slow, short, and dysfunctional in their ability to find ovules, the underlying signaling defects are not known. However, recent evidence culminating from a genetic suppressor screen suggests that CPK17/34 can regulate processes related to cell wall biogenesis. A cpk17/34 suppressor (sc34-1) was identified by screening for mutations that reversed the near-sterile phenotype and resulted in longer siliques with an increased number of seed. Confirming the identity sc34-1 revealed that cpk17/34 can be partially suppressed by null alleles of tbl14 (TBL14 = Trichome Birefringence-Like protein, isoform 14, At5g64020). TBL14 belongs to a family of 46 related proteins in Arabidopsis. Members of this family are proposed to function in the biosynthesis of acetylated cell wall polysaccharides, and thereby influence structural interactions within the cell wall. The discovery that tbl14 can suppress cpk17/34 suggests a working model in which the slow growth of pollen tubes caused by a cpk17/34 KO is at least partly due to a deficiency in cell wall biogenesis (potentially slowing the rate of cell-wall rigidification), which can be reversed by a tbl14 KO that alters cell-wall carbohydrate interactions (potentially accelerating the rate of rigidification). While the exact phospho-regulatory targets of CPK17/34 remain to be determined, the tbl14 suppressor provides genetic evidence for a biological connection between a specific Ca2+-signaling pathway (e.g., CPK17/34) and plant cell wall biogenesis.
Pollen-Pistil Interactions session

Pollen tube sensing of AtLURE1 attractant peptide by tip-localized receptors

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During reproductive process in flowering plants, multistep male-female interactions are essential for successful fertilization. The pollen tube, a tip-growing male gametophyte, is guided by female navigation cues to deliver immotile sperm cells to the egg cell. Pollen tube tip growth is directed by a series of mechanisms called pollen tube guidance. In the final step of the guidance, pollen tubes are precisely attracted by secreted molecules from egg-accompanying synergid cells. We have previously identified species-specific LURE peptides secreted from the synergid cells as key attractant molecules in the final step of pollen tube guidance in dicot plants, Torenia fournieri (Okuda et al., 2009, Nature) and Arabidopsis thaliana (Takeuchi and Higashiyama, 2012, PLoS Biology). LURE peptides induce reorientation of pollen tube tip growth toward the peptides through putative membrane receptor(s) at the pollen tube tip. However, molecular mechanisms and dynamics of LURE reception and signal transduction are unknown.

Here, we report identification of a membrane-spanning receptor kinase essential for directional tip growth toward AtLURE1 attractant peptide in A. thaliana. The receptor kinase is specifically expressed in the pollen tube and localized at the tip of the growing pollen tube. Intracellular domain of the receptor kinase interacted with central regulatory molecules involved in pollen tube growth. Genetic analysis showed that the receptor and related receptors cooperatively recognize AtLURE1 and function in efficient growth and guidance. Additionally, heterologous expression of the receptor in the related species Capsella rubella conferred AtLURE1 responsiveness, suggesting that it acts as a key receptor for recognition of species-specific AtLURE1 peptide. This work provides a molecular basis for directional control of pollen tube growth, which is mediated by tip-localized receptors during male-female communication.
**Pollen-Pistil Interactions session**

**ARC1 is a downstream signalling component of SRK in the self-incompatibility pathway in Arabidopsis spp.**

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Pollen-pistil interactions in flowering plants are tightly regulated to either accept compatible or reject self-pollen. In the Brassicaceae, the rejection of self-pollen is regulated by a signaling pathway activated by the stigma-specific S Receptor Kinase (SRK), following binding of a pollen-specific ligand, SCR/SP11. In Brassica species, downstream signaling components of the pathway have been identified such as the M Locus Protein Kinase and the ARC1 E3 ubiquitin ligase which targets the Exo70A1 subunit of the exocyst complex. While the functions of SCR/SP11 and SRK are conserved in various Arabidopsis species, the downstream signaling pathway leading to the rejection of self-pollen is less clear. We performed a genomic survey of numerous species in the Brassicaceae and determined that ARC1 is frequently deleted in self-compatible species (even though some species still had a functional SRK), indicating that ARC1 may have a conserved role in self-incompatibility signaling in the Brassicaceae. We identified an A. lyrata ARC1 homologue to Brassica ARC1, and investigated if the role of ARC1 is conserved in regulating pollen rejection in the naturally occurring Arabidopsis lyrata self-incompatibility system. We demonstrated that ARC1 was required for self-incompatibility in A. lyrata and have now shifted focus to testing ARC1 in the artificial A. thaliana self-incompatibility system. The expression of SCR/SP11-SRK-ARC1 in A. thaliana resulted in robust self-incompatibility both at the pollen-pistil level and at the cellular level. Currently, the mechanism that underlies the acceptance of compatible pollen grains less well understood, with the exocyst complex recently being demonstrated that it is necessary for the acceptance of compatible pollen grains. To identify additional components of compatible pollination, we are performing proteomics experiments in Brassica rapa.
Variation in interspecific unilateral incompatibility in Arabidopsis

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Gaining a mechanistic understanding of breeding barriers that operate between flowering plants is essential not only for understanding the evolution of breeding systems but also for the transfer of valuable agronomic traits between incompatible species. Previous investigations into interspecific pollination relationships between members of the Brassicaceae have revealed that they often exhibit unilateral incompatibility (UI), i.e. the pollen from the self-compatible (SC) species is inhibited by the pistil from the self-incompatible (SI) species, whereas the reciprocal cross is compatible. This UI phenomenon has been explained by postulating that SI and UI may operate through a shared mechanism, however, the mechanisms underlying interspecific incompatibility are surprisingly poorly understood. In this study, a screen has been carried out to detect variation for interspecific compatibility between A. thaliana accessions and populations of its close SI relatives A. lyrata and A. arenosa with the aim of developing lines to aid in identifying factors that regulate this breeding barrier. The results have revealed that significant variation exists with Arabidopsis on both the male and female side of interspecific pollen-pistil compatibility. Further study has shown that pollen coat carries factors that regulate the interspecific pollen-pistil interaction – for instance incompatibility can be overcome by applying fresh isolated Brassica pollen coat in normally incompatible crosses of ♀ B.oleracea × ♂ A.thaliana. Moreover, the inhibitors cycloheximide and okadaic acid, which are known to effect breakdown of SI in the Brassicaceae, failed to overcome interspecific incompatibility suggesting that the molecular basis of SI and interspecific incompatibility may be distinct. Finally, according to these findings, a new model based on compatibility is proposed for interspecific UI in the Brassicaceae.
Pollen-Pistil Interactions session

Reproductive biology of Chrysanthemum cinerariifolium Vis. (Asteraceae)

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The Dalmatian pyrethrum, Chrysanthemum cinerariifolium, is an herbaceous perennial native to the eastern coast of the Adriatic Sea. Its flowers (particularly the seed trichomes) yield sustainable, natural or “organic” insecticide compounds, particularly Py I, II, that are non-toxic to mammals. Chrysanthemum cinerariifolium possesses a sporophytic self incompatibility system. Our research objectives were to determine a) SI expression in pyrethrum, including pollen germination, pollen tube growth, site of pollen tube arrest, b) time (duration) of pollen tube growth from pollination to fertilization in pollinations to determine embryo rescue at heart stage timing and c) male/female fertility levels. For SI studies, outcrosses and self pollination sets were conducted using select pyrethrum parents with high Py I, II content and high female fertility from composite bulk crosses. After emasculation, pollen was applied to the stigmas and styles were collected from pollinated flowers 6, 12, 18, 24, and 30 hours post-pollination. Styles were fixed in FFA until analyses whereupon they were softened in NaOH and stained with decolorized aniline blue. Under a fluorescence excitation filter, squashed stigmas and styles were observed to detect pollen germination, pollen tube growth in the stigma (site of the SI reaction) and/or pollen tube growth down the style. For male and female fertility levels, pollen stainability and mass or open-pollinated (OP) seed set were used, respectively. Male (MCC) and female (FCC) Coefficients of Crossability were calculated; more than 800 genotypes from seven seed lots (6-commercial, 1-wild population) were assessed. SI plants had pollen germination and pollen tube growth only in stigmatic papillae cells; pollen germination occurred within 6 hrs post-pollination. In some genotypes, pollen tubes reached the stylar base within 6-12 hrs post-pollination while other took >30 hrs. Several plants had low levels of seed set (pseudo self-compatible or PSC) while most were SI. Male fertility (pollen stainability) ranged from 18.7% to 100% while only 18 plants had <50% stainable pollen (the standard cutoff for male sterility); seed lots were significantly different (p=0.002). OP and mass seed set ranged from 0 to 200 and 0 to 322 seeds/inflorescence, respectively. Linear regression of FCC/MCC values for outcross pollinations differed significantly from 1.0; regression with the male- and female-sterile values included was r=0.116. When self seed set data were added, r values increased to match an FCC/MCC distribution similar to other sporophytic SI systems. PSC parents will be useful for generating inbred parents while SI plants could be used for F1 hybrid seed production once trait heritability has been established.
Glutamate receptors in the pollen of Arabidopsis thaliana - On the calcium branch

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The genome of Arabidopsis thaliana contains 20 genes homologous to mammalian ionotropic Glutamate Receptors (iGluRs), denominated Glutamate Receptor-like genes (GLRs). Plant GLRs group into three clades, are able to oligomerize and show a broad, overlapping expression pattern with no obvious preferential tissue expression. Accordingly, functional redundancy and genetic compensation are likely to occur. Indeed, we found that inactivation of multiple GLR genes often only causes mild macroscopic phenotypes. We previously showed that GLRs are Ca2+ channels with important implications in plant reproduction.

Using Arabidopsis pollen as our model cell system and focusing on pollen-abundant AtGLRs, we were able to further describe a variety of in-vitro phenotypes, ranging from branching pollen tubes, slower tube growth and decreased Ca2+ fluxes. In order to better understand the regulation of GLRs, we characterized a group of GLR-interacting proteins with homologies to the Drosophila cornichon (CNI) protein. Our data show that these proteins are not only able to alter the sub-cellular localization of AtGLRs, but can also modify their electrophysiological properties, opening a new angle for the characterization of GLRs.
Variability in the reproductive biology of the wild tomato species S. habrochaites in Ecuador

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Many crop species, including tomato, have such reduced genetic diversity that traditional plant breeding is limited in its ability to achieve crop improvement. The introgression of germplasm from closely related wild species into tomato cultivars has greatly increased yields by introducing resistance to abiotic and biotic stresses. The northern margin of one such wild tomato relative, Solanum habrochaites, is located in southern-central Ecuador. We studied recently collected wild germplasm of Ecuadorean S. habrochaites and analyzed reproductive characters of these populations. We also identified a specific S-RNase allele associated with the loss of self-incompatibility in some populations. These data support the differentiation of four reproductively distinct ecogeographic groups in Ecuador: the central coast (67-200m), central mountains (1250 – 2500m), southern mountains (1600 - 2500m), and southwestern uplands (500 – 1700m). Each group is further delimited by their unique assembly of reproductive features; the southwestern uplands are self-incompatible (SI) while the other groups are all self-compatible (SC). In these SC groups we observed differences in flower size and stigma exsertion. We noted large-flowers and well exserted stigmas in the southern mountains and pale, small flowers in the central coastal region. The central mountains represent a transition region in which stigmas are less exserted than their southern counterparts but are not as extremely reduced as those of the coastal region. We hypothesize this transition may be due to the increased potential for range expansion by reproductive assurance according to Baker’s Law, but further analysis is required. Further, we observed a partial reproductive barrier between populations, in that pollen from some SC populations is rejected by pistils of the SI populations.